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STUDIES ON THE SOIL-INHABITING TARDIGRADE, *MACROBIOTUS* CF. *PSEUDOHUFELANDI*, FROM SOUTH AUSTRALIA

by ALAN F. BIRD *

Summary

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A tardigrade isolated from agricultural soils at Avon is the first member of this phylum to be described from South Australia. Specimens were isolated from freshly-collected soils and from soil that had been stored dry for three years.

Live and fixed specimens were examined under the light microscope and fixed, stained and gold-coated specimens were examined using the scanning electron microscope.

This tardigrade, a stout cylindrical organism about 500 µm long by 150 µm wide with the four pairs of stubby legs ending in paired claws, has been assigned to the species *Macrobiotus* cf. *pseudohufelandi* on the basis of the morphology of the buccopharyngeal apparatus, claw shapes and egg processes. The stylets are slightly curved, sabre-shaped structures about 40 µm in length and exhibit marked birefringence under polarized light. When these tardigrades are killed the stylets break down and disappear.

A specimen of *M.* cf. *pseudohufelandi* was observed feeding on a nematode and a significant decrease in the number of nematodes in the soil as the number of tardigrades increases has been demonstrated.

KEY WORDS: *Macrobiotus* cf. *pseudohufelandi*, anhydrobiosis, microscopy, tardigrades, birefringence, biocontrol.

Introduction

The tardigrades or water bears belong to a discrete phylum, Tardigrada, of cosmopolitan distribution from diverse habitats including marine, fresh water and terrestrial environments. The majority are thought to live in water films surrounding the "leaves" of mosses and lichens. They are microscopic (with adults commonly ranging in length from 200 - 500 µm), are plump and cylindrical in shape and have four pairs of stubby legs ending in claws. They may or may not have eye spots.

In Australia tardigrades have been recorded from Queensland, New South Wales, Victoria, Western Australia and Tasmania but not from South Australia (McInnes 1994).

In this paper I report upon the morphology and some aspects of the behaviour of a tardigrade isolated from agricultural sandy loam soil from Avon, South Australia.

Materials and Methods

Locality and soil type

The tardigrades were isolated from a soil of sandy loam texture classified as a solonized brown earth (Australian soil grouping) or as an entisol (US soil classification). The locality was an experimental plot on a farm at Avon (latitude 34° 14' S , longitude 138°19' E) which was direct drilled and had a wheat/wheat rotation.

Soil cores (diam. 5 cm, depth 10 cm) were collected and mixed in a plastic bag. The sample mostly used in these experiments was collected in July 1993 and had been stored dry at room temperature for three years. However, freshly-collected soil from the same site on 29. iii. 1996 prior to the autumn rains was also used for comparison.

Extraction from soil

After thorough mixing of the soil, 50 g aliquots were placed in a misting apparatus for three days (Yeates & Bird 1994). This procedure was replicated in quadruplicate and, after three days, the collecting tubes were removed and their contents allowed to settle for 1 h after which the supernatant was removed by suction to within 2.5 cm of the bottom of the tube. This extraction procedure was used for all soils, whether freshly-collected or stored.

Counting

The contents of each tube were poured, after vigorous shaking, into a counting chamber (Doncaster 1962). The tardigrades gravitated to the floor of the counting chamber between the rings and were counted under a dissecting microscope.

Light microscopy

The tardigrades were examined under bright field, polarized light and differential interference contrast (Nomarski) optics using a Vanox Olympus AHBT research microscope.

Living tardigrades were examined in distilled water under a coverslip sealed at its edges with nail varnish.

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Specimens were fixed by adding an equal volume of boiling double strength FA 4:1 (20 ml 40% formaldehyde and 2 ml glacial acetic acid in 78 ml distilled water) in a test tube to a shaken suspension of the tardigrades in distilled water, also in a test tube. These specimens were processed to pure glycerol by Seinhorst's (1959) method and mounted in anhydrous glycerol on slides sealed to a coverslip by molten paraffin as described by De Maeseneer & D'Herde (1963) and then ringed with Entellan (Merck). Both living and fixed material were photographed using Ilford Delta 400 film.

Scanning electron microscopy

For observations under the scanning electron microscope (SEM), the fixed material was washed repeatedly in distilled water, post-fixed and stained in 1% aqueous osmium tetroxide, washed repeatedly again in distilled water, immersed in filtered freshly-made saturated aqueous thiocarbohydrazide for 30 min followed by repeated washings in distilled water and a repetition of the osmium fixation. This osmium-binding technique (Kelley *et al.* 1973) was followed by further washings in distilled water.

Specimens were freeze-dried by placing them between membrane filters which were frozen rapidly by placing them in a slurry of freon cooled by liquid nitrogen. The filters with attached tardigrades were then transferred rapidly to a freeze drying machine and freeze-dried at -70° C over a period of three days. This dried material was then mounted on a glass coverslip attached to an SEM stub and coated with 30 nm of gold to enhance stability and conductivity. The material was then examined and photographed in a Cambridge S 250 Mk 3 SEM operated at 20 kV using Ilford 120 roll film (FP4 Plus).

Feeding experiments

Attempts to determine whether or not *M. cf. pseudohufelandi* would feed on *Rhizoctonia solani*, and thus possibly implicating the tardigrade in suppression of this plant pathogen in the field, were made using *R. solani* grown in culture media in Petri dishes. Specimens of *M. cf. pseudohufelandi* that had been washed repeatedly in sterile distilled water by centrifugation in an effort to surface sterilize them, were pipetted on to the fungal plates under aseptic conditions in a laminar flow cabinet.

Results

Morphology

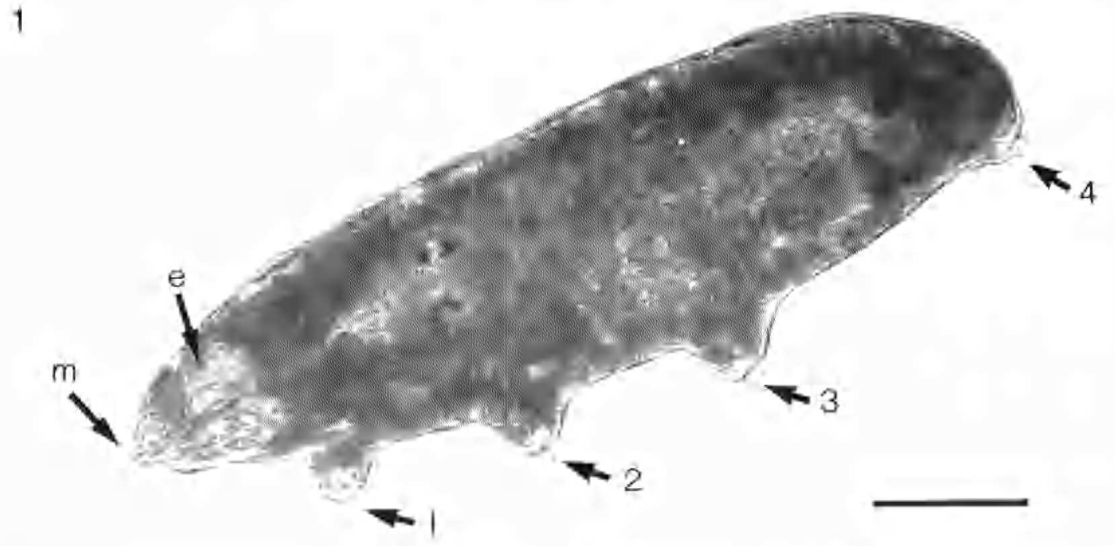
Both living and fixed specimens of the tardigrade isolated from the Avon soil were examined and measured. Measurements of the lengths and widths of ten specimens each of fixed and living material showed, as might be expected, that some shrinkage had occurred in the fixed material. Measurements of the living material were made only on specimens that had their lips and mouth parts retracted since this was the state observed in all fixed or dead material. The mean length of living specimens was 511.4 ± 47.7 µm (range 428-580 µm, Table 1) and that of fixed material was 423.2 ± 48.3 µm (range 361-500 µm). Similarly, the width of the living specimens was 154.7 ± 15.3 µm (range 128-172 µm, Table 1) compared with 131.2 ± 13.3 µm (range 108-148 µm).

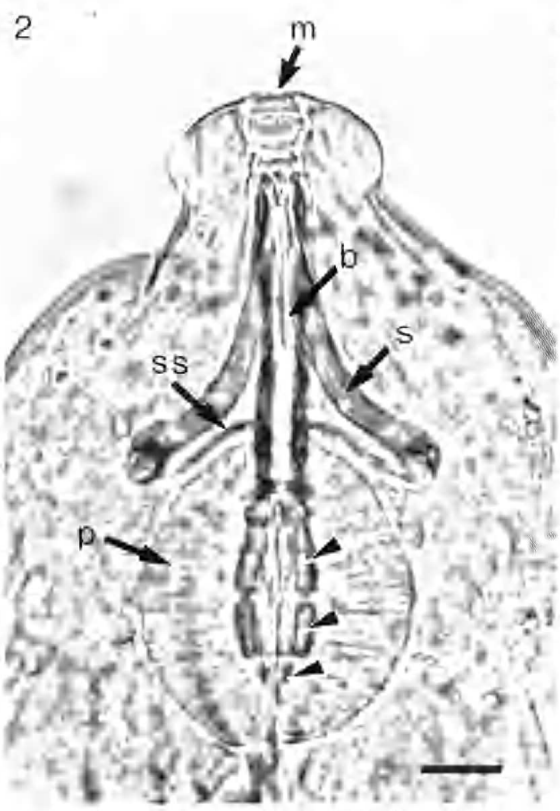
The lateral view of the living tardigrade with its mouth everted and showing one of each of the four pairs of stubby legs ending in claws (Fig. 1) together with the characteristic internal structure of the anterior region (Fig. 2) and the claws at the extremities of the fourth pair of legs (Figs 3, 4) are shown as viewed under the light microscope.

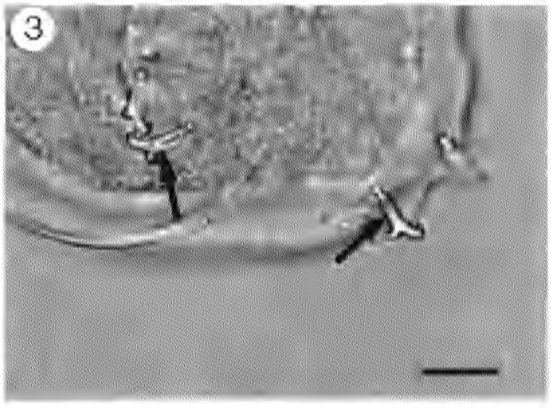
The fixed tardigrade in ventro-lateral view with the mouth region inverted but showing all four pairs of legs with their claws (Fig. 5) and the ultrastructure of the claws on the second leg (Fig. 6) are shown as viewed under the SEM.

TABLE 1. *Measurements of living* Macrobiotus *cf.* pseudohufelandi

Part measured (µm)	Numbers measured	Mean	S.D.	Range
Length of whole specimen	10	511.4	± 47.7	428-580
Width of whole specimen	10	154.7	± 15.3	128-172
% position of stylet support on buccal tube	8	81.0	± 1.3	79.5-82.5
Length of buccal tube	8	40.25	± 1.2	39-42
Width of buccal tube	8	5.1	± 0.2	5-5.5
Length of pharyngeal bulb	8	37.4	± 2.1	35-40
Width of pharyngeal bulb	8	34.4	± 1.3	33-36
Length of macroplacoid (1)	8	10.75	± 1.0	9-12
Length of macroplacoid (2)	8	6.0	± 0.5	5-7
Length of microplacoid	8	2.9	± 0.2	2.5-3.0
Length of 4th foot claw	4	8.25	± 2.9	8-9







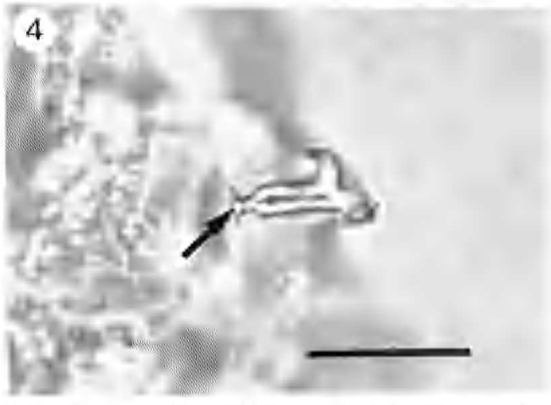


Fig. 1. Whole living *Macrobiotus* cf. *pseudohufelandi*. Bright field optics. Lateral view showing everted sucking mouth (m), eyespot (e) and one of each of the four pairs of legs with terminal claws (arrows labelled 1-4 from front to rear). The fourth leg is posterior and subterminal. Scale bar = 100 µm.

Fig. 2. Everted head of living *Macrobiotus* cf. *pseudohufelandi*. Bright field optics showing mouth (m), buccal tube (b), stylets (s), stylet support (ss), pharynx (p), macroplacoids 1 & 2 and microplacoids (arrowheads from top to bottom). Scale bar = 10 µm.

Fig. 3. Posterior region of *Macrobiotus* cf. *pseudohufelandi*. Bright field optics showing claws of the 4th legs (arrows). Scale bar = 10 µm.

Fig. 4. Single claw from the 4th leg of *Macrobiotus* cf. *pseudohufelandi*. Note basal plate at base of claw (arrow). Scale bar = 10 µm.


5
1
2
3
4


6

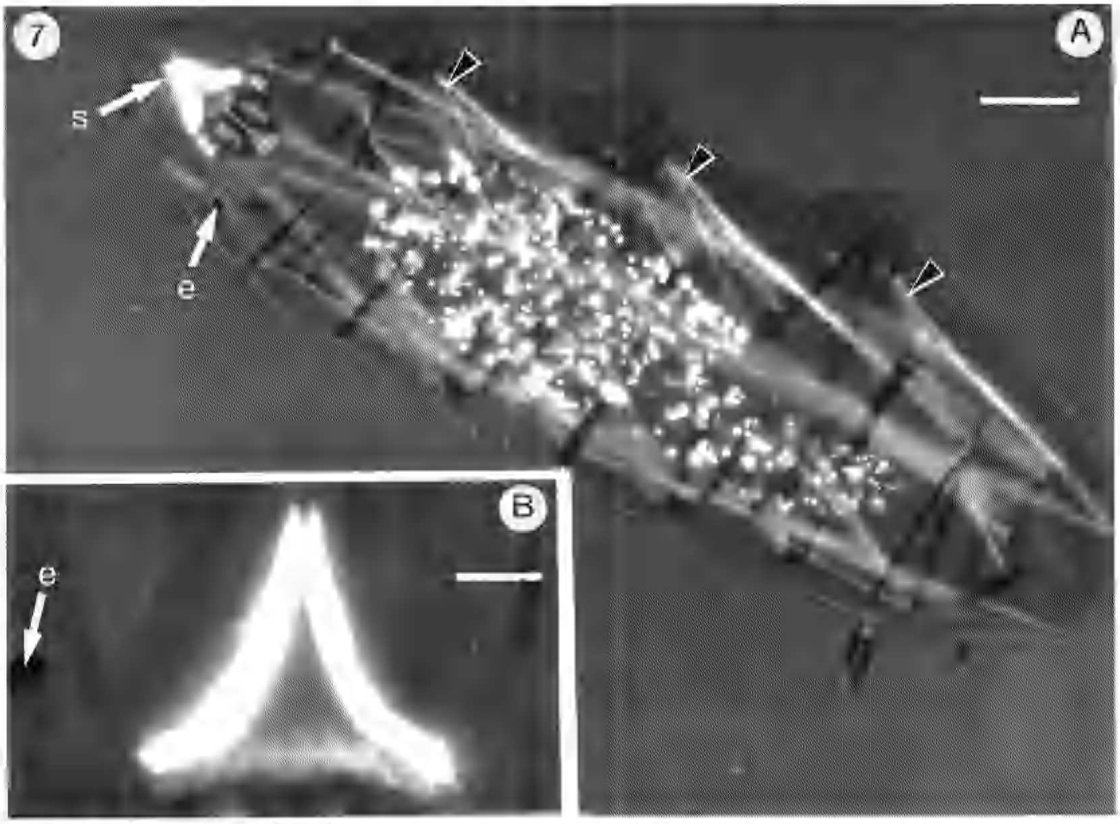


Fig. 7. Living *Macrobiotus* cf. *pseudohufelandi* viewed under polarized light. A. Whole specimen. Note birefringence of stylets (s), gut contents and muscle attachments to legs (arrow heads). One of the two eyes (e) is also shown. B. Inset showing an eye (e) and the pronounced birefringence of the two stylets. Scale bars = 50 μm A, 10 μm B.

Although for general purposes bright field optics were found to be more convenient than the other systems used, stylet structure was much more obvious in living tardigrades under Nomarski and polarized light optics than under bright field. Stylets were, however, clearly observed under all three optical systems provided that the tardigrade was alive. In both dead and fixed material, the stylets lose their integrity and appear to break down.

When viewed under polarized light (Fig. 7) the stylets exhibited marked birefringence indicating a regular structural orientation. The stylets are slightly curved, sabre-shaped structures about 40 μm in length (Figs 2, 7B). When the tardigrades were subjected to oxygen deprivation on sealed slides, the stylet birefringence gradually disappeared and was entirely lost over a period of several hours as the stylets broke down. Muscles and intestinal contents exhibited birefringence to a lesser extent than the stylets (Fig. 7). However, the muscles and intestinal contents retained their integrity and birefringence after stylet break down. Stylet break down also occurred during fixation which accounts for their absence in camera lucida drawings of fixed material in the literature. Thus stylets can only be observed in living material.

The buccal tube (Fig. 2, Table 1) is 40.25 μm long x 5.1 μm wide (percent ratio to the length of the buccal tube (pt) = 12.67). The stylet supports (Fig. 2, Table 1) are inserted at 81% of the buccal tube

Fig. 5. Whole, fixed and gold-coated specimen of *Macrobiotus* cf. *pseudohufelandi*. SEM photograph showing four pairs of legs with claws (arrows labelled 1-4 from front to rear). Scale bar = 100 μm.

Fig. 6. SEM photograph of a 2nd leg. Note two sets of double branched claws. The inner branch in each claw carries two accessory points (small arrows). The basal plate at the base of the claw is seen here as a rounded thickening (larger arrow). Scale bar = 4 μm.

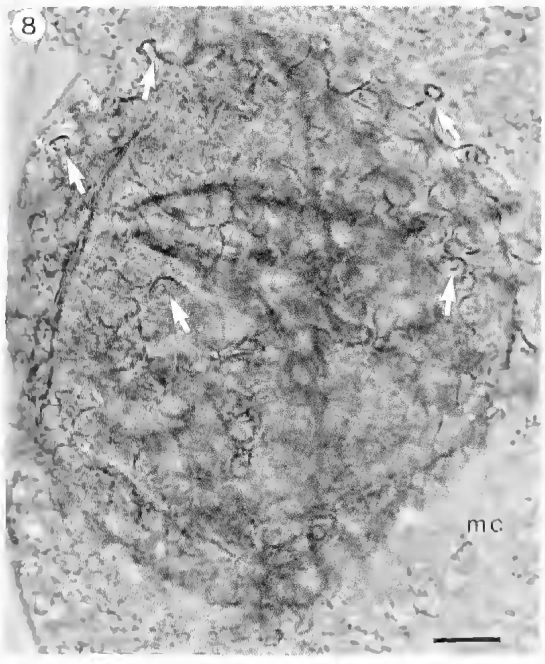


Fig. 8 Crushed egg of *Macrobiotus* cf. *pseudohufelandi* lying within the moulted cuticle (mc). Bright field optics showing the "cooling tower"-shaped projections on the surface of the egg shell (arrows). Scale bar = 10 µm.

length. The pharyngeal bulb is 37.4 µm long x 34.4 µm wide, has apophyses, two pairs of rod-shaped macroplacoids and a pair of microplacoids. The macroplacoids differ in length, the first being 10.75 µm long (pt = 26.71) and the second 6.0 µm (pt = 14.91); the microplacoids are 2.9 µm long (pt = 7.21). The claws (Figs 3, 4, 5, 6, Table 1) have smooth basal plates (Dastych & Alberti 1990) and well-developed accessory points on their inner branches. The length of the fourth foot claw is 8.25 µm (pt = 20.50). The egg, enveloped by the shed cuticle, (Fig. 8) has numerous "cooling tower"-shaped projections on its surface which protrude about 5 µm.

Taxonomy

This tardigrade was, using available keys and on comparison of measurements, determined as *Macrobiotus hufelandi* Schultze, 1834. This species is cosmopolitan (Schuster & Grigarick 1965). However, measurements and slides were sent to a tardigrade taxonomist (S. Claxton) who kindly examined the material and considers that this tardigrade most closely resembles *Macrobiotus pseudohufelandi* Iharos, 1966 and should be assigned tentatively to this species (S. Claxton pers. comm.). Accordingly, the Avon tardigrade has been identified as *Macrobiotus* cf. *pseudohufelandi* subject to further deliberations by taxonomists.

Behaviour

Specimens of *M.* cf. *pseudohufelandi* were placed in Petri dishes containing the plant pathogenic fungus *Rhizoctonia solani*. Although some of the tardigrades lived for several weeks, they were not observed to feed, grow or reproduce on the fungus. Furthermore, no significant differences were observed in the logarithmically transformed numbers of this tardigrade isolated from soil that exhibited suppression of this fungus compared with the tardigrade numbers, logarithmically transformed, isolated from soil that did not exhibit suppression of the fungus ($p < 0.05$) (Table 2). However, there does appear to be a significant inverse linear relationship between the numbers of *M.* cf. *pseudohufelandi* in the soil and the numbers of nematodes ($p < 0.001$). Furthermore, a specimen has been obtained alive from the soil that was in the process of feeding on a nematode (Fig. 9). In soil that had been stored dry for three years, there was a marked decline in the numbers of nematodes (4 ± 2.9 /50 g soil) compared with the number of tardigrades (34 ± 5.8 /50 g soil).

Discussion

The first recorded observation of a tardigrade was by Goeze (1773) (cited by Nelson & Higgins 1990) who referred to them as "little water bears" (*Kleiner*

TABLE 2. *The relationships between tardigrades and nematodes in* Rhizoctonia-*suppressive and* Rhizoctonia-*non-suppressive soils*

Soils	Tardigrades		Nematodes		Tardigrades	Nematodes
	Mean counts*	SD	Mean counts*	SD	log mean counts†	log mean counts†
Suppressive	19	± 9.1	401	± 108	2.81	5.97
Non-suppressive	32	± 4.8	227	± 149	3.44	5.25

*SED = 65.2

† SED = 0.346
† LSD = 0.75

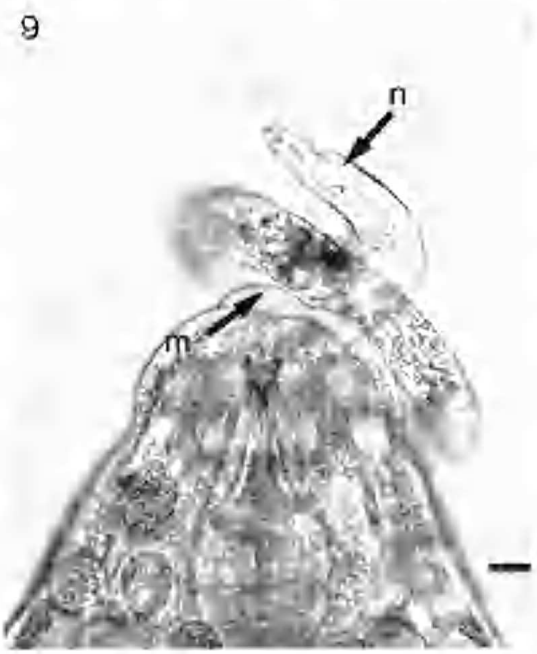


Fig. 9. Head region of a living *Macrobiotus* cf. *pseudohufelandi*. Photographed in the process of feeding on a nematode. Bright field optics showing mouth (m) and nematode (n). Scale bar = 10 µm.

Wasser Bär). Three years later, Spallanzani (1776) (cited by Nelson & Higgins 1990) called them "slow steppers" or *Il Tardigrado* which gave rise to the phylum name used today. Because of their characteristic morphology, the tardigrades are recognized today as belonging to a discrete phylum. They probably evolved more than 500 million years ago in the Cambrian period when there was an explosive diversification of eukaryotic organisms. Although the Avon tardigrade clearly belongs to the *hufelandi* grouping in the genus *Macrobiotus* (Bertolani & Rebecchi 1993) its specific taxonomic identity is subject to further deliberation. This tardigrade has similar measurements to a population of *M.* cf. *pseudohufelandi* that includes specimens from WA (S. Claxton pers. comm.). Thus, despite the fact that they have "cooling tower"- shaped egg shell projections that are more like those illustrated for *M. hufelandi* (Nelson & Higgins 1990) than those illustrated for *M. pseudohufelandi* by Ramazzotti & Maucci (1983), which have much broader bases, they are tentatively assigned to *M.* cf. *pseudohufelondi* (S. Claxton pers. comm.).

The ability of tardigrades to survive in a wide range of environments and their world wide dispersal must be due in no small way to their ability to enter into an anhydrobiotic state, a function that they share with some nematodes and rotifers. I have shown that *M.* cf. *pseudohufelandi* can survive for at least three years in dry soil maintained at room temperature. This was the maximum time tested and it seems likely that these creatures could survive for much longer under these conditions, since it has been reported (Keilin 1959) that survival times of up to 10 years can occur. Indeed, recovery, but not survival, has been reported to occur after 120 years of anhydrobiosis (Francheschi 1948 cited by Crowe 1971).

Anhydrobiosis is widespread in Phylum Tardigrada and it is thought that the disaccharide trehelose functions to protect these organisms since it accumulates within them as they are exposed to desiccation (Westh & Ramløv 1991). In this respect tardigrades resemble those nematodes that exhibit anhydrobiosis (Madin & Crowe 1975). In their morphology, of course, they are completely different and this is reflected in their different behaviour when observed in water under a coverslip. For example, *M.* cf. *pseudohufelandi* is able to push its way through an air bubble. This is a capability that I have never observed in a nematode.

The composition of the two sabre-shaped stylets does not appear to have been studied in detail and they are absent from camera lucida drawings because they break down and disappear in fixatives. Similarly, they break down on the natural death of the animals caused by anoxia on sealed slides. It seems strange that structures which, in the course of predation and feeding, can penetrate both plant and animal tissues, should be so fragile.

In the living state, the stylets are readily observed under all optical systems. However, when viewed under polarized light they exhibit marked birefringence indicating a regular structural orientation. It has been mentioned in the literature that the stylets are calcareous (Kaestner 1968) so their birefringence is probably crystalline and their break down under anoxic conditions might be due to an increased internal acidity leading to the dissolution of calcareous structures such as the stylets. Clearly such an hypothesis requires further testing.

Macrobiotus cf. *pseudohufelandi* was not observed to feed on the plant pathogenic fungus *Rhizoctonia solani* and I conclude that the tardigrade which I have found in large numbers in agricultural soils in South Australia probably does not feed on this fungus in the field but preys on other small soil organisms such as nematodes.

The ability of tardigrades to prey on nematodes has been recognized for some time. In 1969 Sayre showed that the tardigrade *Hypsibius myrops* could be cultured using the free-living nematode *Panagrellus redivivus* as prey (Sayre 1969). Furthermore, Sayre (1969) showed that *H. myrops*

was able to feed on the plant parasitic nematodes *Meloidogyne incognita* and *Ditylenchus dipsaci*. Sayre (1969) concluded that "under certain circumstances, (tardigrades may) give some control and these need to be investigated". A major draw back to this work was that it was conducted in a moist environment using moss as a substrate. This is a far cry from the normal environment of the plant parasitic nematodes that Sayre used in his experiments.

The tardigrade that I have reported upon in this paper comes from a true agricultural soil environment where it has to survive extremely harsh and variable conditions. Under these conditions, it may have the capacity to reduce nematode populations although the decline in the numbers of nematodes compared with the number of tardigrades may reflect the anhydrobiotic capabilities of the tardigrades compared with those of the mixed nematode population rather than predation by the tardigrades. However, *M.* cf. *pseudohufelandi* may be an effective biocontrol agent, although little is known of its nematode food preferences and a more quantitative assessment of its potential for biocontrol of plant parasitic nematodes is needed.

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